Electronic supplementary information

Co-Cu bimetallic MOF-derived nanozyme-based colorimetric immunoassay with smartphone-based RGB readout for sensitive detection of alpha-fetoprotein

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Experimental Characterization. Scanning electron microscopy (SEM, HITACHI S-4800, Japan) was used to observe the morphology of the nanozyme. Transmission electron microscopy (TEM, Thermo Fisher Scientific Tecnai G2 F20, USA) and high-resolution TEM (HRTEM) were used to characterize the microstructure and crystal structure of the nanozyme. Energy dispersive X-ray spectroscopy (EDS) mapping (attached to TEM) was used to analyze the elemental distribution of the nanozyme; Electron paramagnetic resonance (EPR, Bruker A300, Germany) was used to detect the structural vacancies of the nanozyme; ultraviolet-visible (UV-Vis) spectrophotometer (Shanghai INESA L6S, China) was used to record the absorbance of the chromogenic system. Enzyme label analyzer (Bio-Rad Model 680, USA) was used to measure the absorbance of the solution in the microplate. Smartphone equipped with self-developed RGB color analysis software was used for the visual readout of colorimetric signals. Analytical balance (Sartorius BSA224S, Germany) was used for weighing reagents; constant temperature water bath oscillator (Shanghai Yiheng SHZ-C, China) was used for incubation reactions. High-speed centrifuge (Hunan Xiangyi H1850R, China) was used for the separation of solid and liquid phases. All volunteers participating in sample collection signed written informed consent forms. The collection, processing, and experimental use of samples strictly adhered to international medical ethical guidelines and relevant Chinese laws and regulations.

Measurement of Enzymatic Activity. The enzyme-like activities of the synthesized nanozymes were performed according to the previous reports.[1] The catalytic reactivity of CCBF-based nanozymes was defined as a unit number of nanozyme reactions yielding 1.0 μmol of product per minute. Briefly, 1000 μg mL^{-1} of nanozymes catalyst dispersion (in NaAc-HAc buffer) was pre-prepared. After that, 189 μL of HAc-NaAc buffer (pH 3.6), 10 μL of TMB solution (10 mg mL^{-1} in DMSO), and 1.0 – 5.0 μL of nanozyme solution were sequentially added to a pre-warmed 96-microwell plate at 37 °C. Thereafter, 3.33 M hydrogen peroxide solution (100 μL) was added and the absorbance change at 652 nm was recorded immediately in a preheated (37 °C) enzyme marker. The change in absorbance obtained was recorded as *s*. The enzyme activity of the nanozyme was calculated according to the following equation (1):

$$b = \frac{V}{\varepsilon l} \times s \quad (1)$$

where V represents the total reaction volume; L represents the optical range; and ε represents the absorbance coefficient (39,000 M s^{-1}). Based on the reaction activity of the enzyme obtained, data

were processed with the total mass (m) of enzyme added to obtain the unit activity of the enzyme according to Equation (2):

$$a = b/m \quad (2)$$

To further determine the steady-state kinetic parameters in the catalyzed reaction, the affinity test of PB-based nanozyme for different concentrations of H_2O_2 substrate was evaluated. Different concentrations of hydrogen peroxide solutions (0.1 mM to 10 mM) were set as experimental groups and a fixed concentration of nanozymes-based catalyst (1 μg) was used for the evaluation of the reaction rate in the experiments according to Eq. 2. In particular, the kinetics constants V_{max} and *K*m was determined by fitting the reaction velocity values and the substrate concentrations to the Michaelis-Menten equation as follows equation (3,4):

$$v = (vmax \times S)/\ （Km + S） \quad (3)$$

Preparation of mAb_1-Coated Microplate. Alpha-fetoprotein (AFP) monoclonal antibodies were immobilized on microplate wells following a standardized coating protocol. A 96-well high-affinity polystyrene microplate was loaded with 50 μL of the purchased capture antibody (mAb_1) at a concentration of 10 μg mL^{-1}, which was diluted in 0.05 M sodium carbonate buffer (pH 9.6). The microplate was then sealed with a plastic film to minimize solvent evaporation and incubated statically at 4 °C for 24 h to facilitate antibody adsorption. After the overnight incubation, the coated wells were subjected to three washing cycles using phosphate-buffered saline containing 0.05% (v/v) Tween 20 (PBST, pH 7.4) to remove unbound antibodies. Subsequently, non-specific binding sites on the microplate surface were blocked by adding 300 μL of blocking buffer, consisting of 10 mM PBS (pH 7.4) supplemented with 1.0 wt % bovine serum albumin (BSA), followed by a 1 h incubation period. The microplates prepared through this process were stored appropriately and used directly for subsequent AFP immunoassays.

Preparation of Detection Antibody-Conjugated GS-CCBF. 3-Amino-1,2,4-triazole (Atz, 48 mg, 0.56 mmol) was added to a suspension of GS-CCBF nanozymes (5 mL, 10 mg mL^{-1}), and the mixture was reacted at 50 °C for 2 h. The resultant product was collected via two successive centrifugation steps (6000 rpm, 5 min for each) and re-dispersed in 1 mL of deionized water. For the EDC/NHS activation process, the reaction system was maintained at pH 7.4 and the reaction was conducted at 37 °C. The AFP antibody (0.25 mg mL^{-1}) was incubated with EDC (5.1 mg, 100 μL) for 30 min, and then NHS (7.7 mg, 100 μL) was added for another 30-min incubation to obtain NHS-

activated AFP antibody. This activated antibody was then mixed with the CCBF-NH_2 dispersion and subjected to a 2 h incubation.[2] The final product was harvested by centrifugation and resuspended in 1 mL of deionized water.

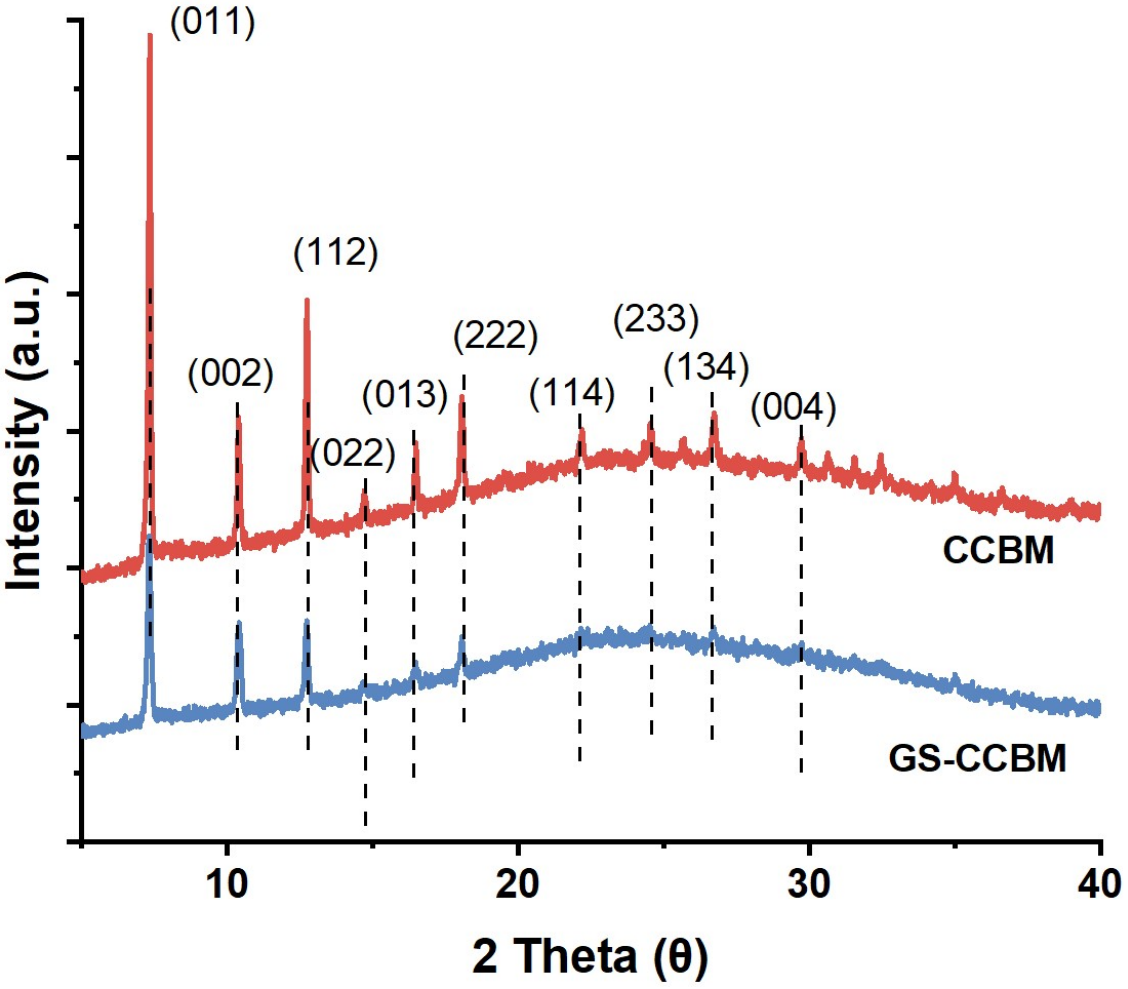

Figure S1. X-ray diffraction patterns of synthesized nanozymes.

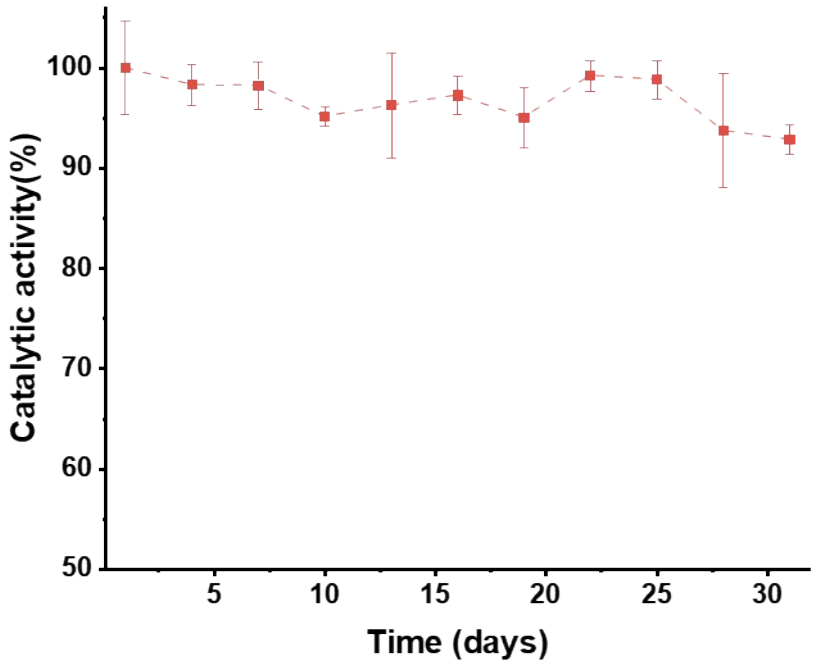

Figure S2. Catalytic activity of synthetic GS-CCBM nanozymes under different storage conditions.

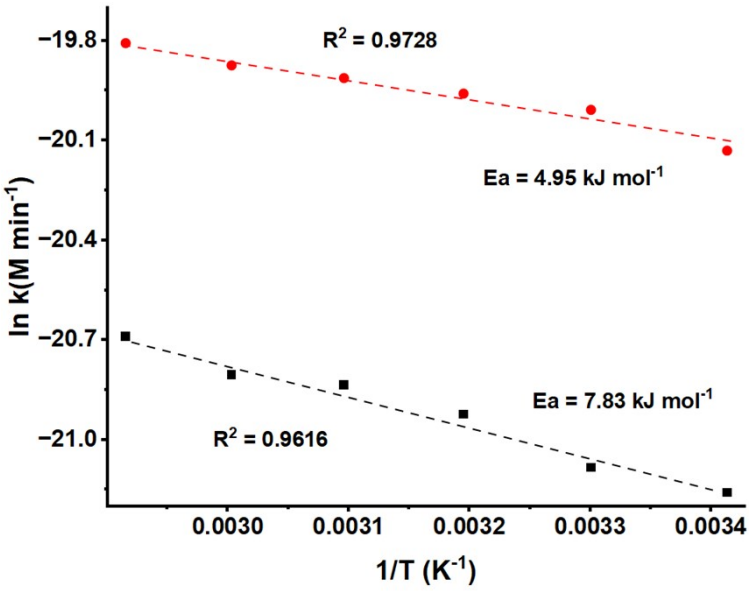

Figure S3. Thermodynamic Arrhenius curves for the hydrogen peroxide reaction catalyzed by CCBF nanozymes (black) and GS-CCBF nanozymes (red).

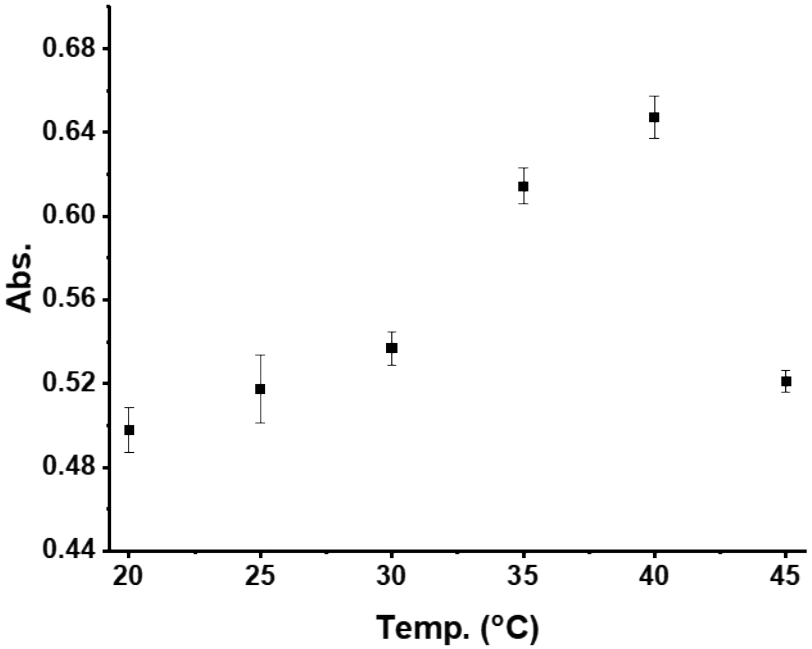

Figure S4. Systemic absorbance variation curve of AFP target (0.1 ng mL^{-1}) under different incubation temperatures.

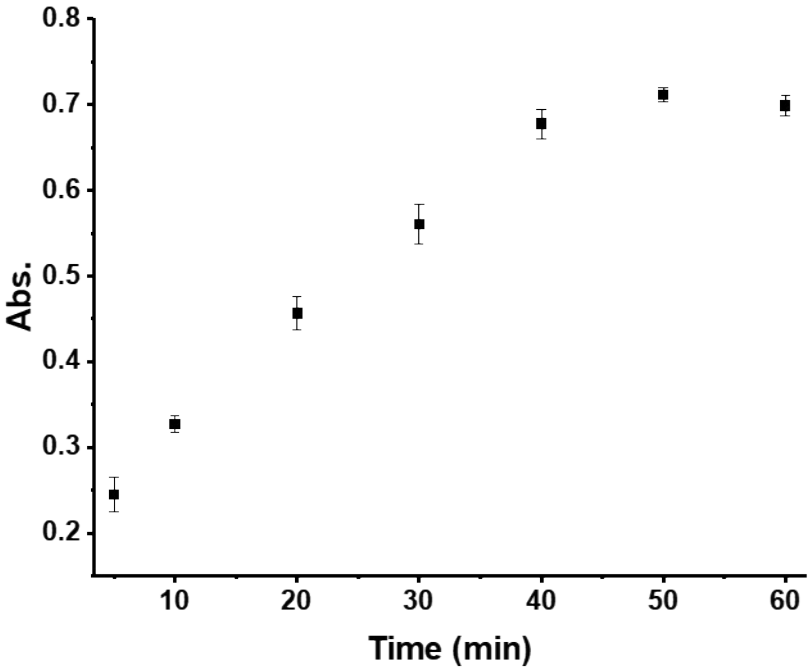

Figure S5. Systematic absorbance variation curves of standard AFP samples under different catalytic time conditions. Results indicated that absorbance tends to stabilize when catalytic time exceeds 40 min.

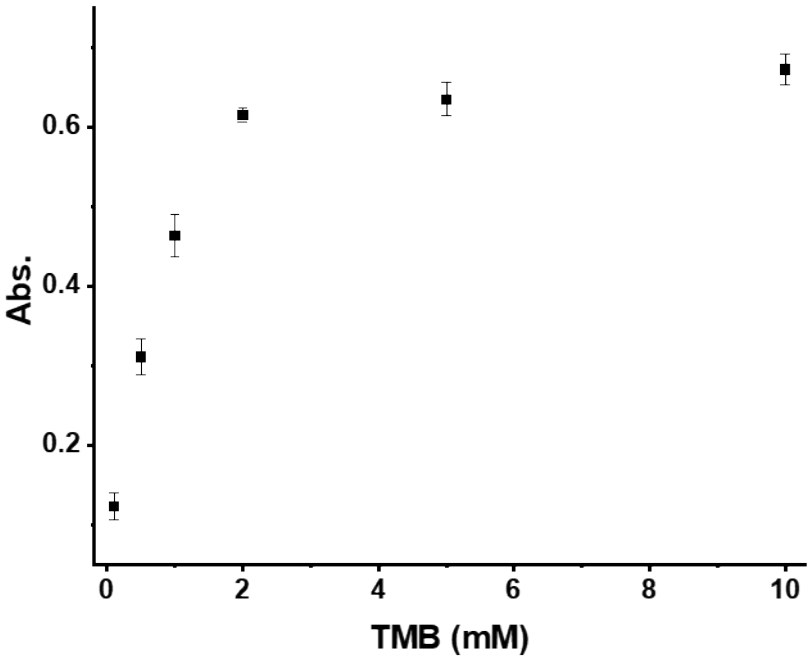

Figure S6. Based on the trend of changes, we selected a TMB concentration of 5 mM as the final reaction catalytic condition.

Table S1. Table comparing the reaction constants of synthesized nanozyme catalysts with those of developed nanozymes.

Materials	Km (mmol)	Vm (μmol min^{-1})	SA (U mg^{-1})
Au@Pt[3]	0.231	1.96	27.2
MO@MOFs[4]	0.522	1.26	4.98
FeN_3S[5]	0.67	0.61	26.82
Fe_2N_7[6]	3.36	0.07	24.7
FeCu–P/carbon dots[7]	0.27	4.26	37.9
Ce-MOF[8]	0.012	0.112	0.97
PtCo[9]	-	-	3.042
Prussian blue[10]	12.3	0.48	-
CCBM	3.232	2.349	0.98
GS-CCBM	1.79	8.569	6.19

Table S2. Comparison table of dynamic response range and LOD values for AFP target detection methods.

Detection method	Dynamic range	LOD	Target
lateral flow immunoassay[11]	5-300 ng mL^{-1}	0.768 ng mL^{-1}	CEA
ELISA[12]	0.2 - 80 ng mL^{-1}	0.15 ng mL^{-1}	CEA
lateral flow immunoassay[13]	5 - 200 ng mL^{-1}	0.19 ng mL^{-1}	AFP
Electrochemical[14]	0.2 - 100 ng mL^{-1}	74.8 pg mL^{-1}	AFP
Paper-based ELISA[15]	0.5 - 60 ng mL^{-1}	136 pg mL^{-1}	CEA
NLISA	0.01 - 50 ng mL^{-1}	4.7 pg mL^{-1}	This work

Table S3. Comparison of paper-based pec sensing method with ELISA kit for AFP in real sample analysis.

Simple no.	Methods (mean ± SD, ng mL^{-1}, n = 3)		t_{exp}
	Developed Method	ELISA kit	
1	4.59 ± 0.25	4.55 ± 0.19	0.77
2	0.97 ± 0.08	1.18 ± 0.13	2.14
3	0.06 ± 0.009	0.072 ± 0.006	0.71
4	0.38 ± 0.04	0.41 ± 0.016	0.24
5	11.32 ± 0.57	11.96 ± 0.89	0.77
6	6.51 ± 0.27	6.80 ± 0.37	0.86
7	2.86 ± 0.09	2.61 ± 0.14	1.07
8	0.098 ± 0.014	0.13 ± 0.025	1.77

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